Seroprevalence of *Toxoplasma gondii* in southern districts of Tamil Nadu using IgG-ELISA

G. Sucilathangam · N. Palaniappan ·
C. Sreekumar · T. Anna

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Abstract The present study was conducted to assess the seroprevalence of *Toxoplasma gondii* in and around Tirunelveli by in-house IgG assay using ELISA. Serum samples from 175 immunodeficient and 175 immunocompetent patients were collected at Tirunelveli district, Tamil Nadu from May 2006 to October 2007. They were subjected into in-house IgG assay using enzyme-linked immune sorbent assay (ELISA) in which tachyzoite soluble antigen derived from solubilised whole organisms was used. Out of 350 patients tested by IgG ELISA, 46 patients (13.14%) had antibodies for toxoplasmosis with mean OD value of 0.2 ± 0.073 and the OD value ranged from 0.144 to 0.444. Among the immunocompetent group of 175 patients, 19 patients (10.86%) had antibodies to toxoplasmosis whereas, in immunodeficient group of 175 patients, 27 patients (15.43%) had antibodies for toxoplasmosis. There was no statistical difference ($P > 0.05$) between the immunocompetent and immunodeficient group. The sensitivity and specificity of IgG ELISA in detecting toxoplasmosis was 90 and 100%, respectively. The overall seroprevalence of toxoplasmosis in and around Tirunelveli district of Tamil Nadu was 13.14% based on IgG ELISA. The study has proved ELISA to be a sensitive and specific procedure for the serodiagnosis of toxoplasmosis.

Keywords Seroprevalence · *Toxoplasma gondii* · Enzyme-linked immunosorbent assay (ELISA) · Tamil Nadu

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite and is distributed globally. It was estimated that one-third of the world's human population is exposed to the threat of this parasite (Hughes 1985). *T. gondii* is an opportunistic pathogen, which is generally asymptomatic in the immunocompetent individual. However, the infection of this parasite can result in severe complications or even death in people who are severely immunocompromised, such as patients with neoplastic disease, organ transplantation, and AIDS (Conrath et al. 2003). In pregnant women, the primary infection of *T. gondii* may cause abortion, neonatal malformation, neonatal death, or severe congenital deficiency, such as mental retardation, retinochoroiditis and blindness (Kravetz and Federman 2005). In addition, toxoplasmosis is one of the main causes of foetal abortion, stillbirth, and neonatal mortality in domestic animals, resulting in significant economic loss in the farming industry (McAllister 2005).

Detection of *T. gondii* infection with sensitive and specific methods is a key step to prevent and treat the toxoplasmosis. Clinically, however, the diagnosis of toxoplasmosis is difficult because the clinical signs and symptoms are varied and mimics those of a variety of other diseases (Hurt and Tammam 2007). To date, many diagnostic methods including pathogenic, immunologic, and molecular techniques have been used for detection of *T. gondii* infection; of them, serologic methods such as dye test, indirect hemagglutination test, latex agglutination test,

G. Sucilathangam (✉) · N. Palaniappan
Department of Microbiology, Tirunelveli Medical College,
Tirunelveli 627 011, Tamil Nadu, India
e-mail: drgsucila@rediffmail.com

C. Sreekumar · T. Anna
Department of Veterinary Parasitology,
Tamil Nadu Veterinary and Animal Sciences University,
Chennai 600 051, India

indirect fluorescent antibody test, and enzyme-linked immunosorbent assay (ELISA) are most commonly used (Beghetto et al. 2006; Taylor et al. 1990).

In India, the exact seroprevalence of this infection is not known. However, using various diagnostic tests, the prevalence has been reported to be as low as 1% and as high as 80% in adults (Singh 2003). However, the knowledge about this infection, diagnosis and interpretation of the test results is a major problem in the Indian context. Though, *Toxoplasma* infection does not cause repeated foetal losses, this is the most common indication for investigation of toxoplasmosis in India.

There are several diagnostic test kits available in Indian markets, however, their qualities are not assessed by most of the laboratories before they are procured (Singh et al. 1997). There is no baseline data on seroprevalence and antibody titres of toxoplasmosis in various subpopulations in different parts of our country. In Tamil Nadu, various studies have done in northern districts (Bhatia et al. 1974; Manikandan et al. 2006) but so far not reported in southern districts except our earlier recent report (Sucilathangam et al. 2010). Further complicating the situation, there are several commercial organizations that are promoting their products without proper background knowledge and baseline data from India.

Under this situation, it is highly imperative to assess the status of seroprevalence of toxoplasmosis in southern parts of Tamil Nadu especially in pregnant women and immunodeficient patients by using in-house IgG ELISA and the IgG ELISA test results were compared with our previous report on seroprevalence of toxoplasmosis using same set of samples by IgG IFAT.

Materials and methods

Study population

After obtaining Institutional Ethical Committees approval and informed consent from the patients, a total of 350 peripheral blood samples were collected from 175 immunodeficient patients (HIV and patients with malignancy) and 175 immunocompetent patients including pregnant women (135), ocular chorioretinitis cases (20), and patients with lymphadenopathy (20), in and around Tirunelveli district of Tamil Nadu.

Preparation of tachyzoite soluble antigen (TSA)

As per the procedure outlined in US Department of Health, Education and Welfare Manual (USHDEW 1976) the *T. gondii* (RH strain) tachyzoite antigen was prepared by *in vivo* mice propagation and *in vitro* cell culture system in

Madin Darby Canine Kidney fibroblast (MDCK) cell line. The tachyzoite count was 1×10^7 and 1×10^9 per ml of antigen *in vivo* and *in vitro* methods, respectively.

Purified formalin killed tachyzoites were lysed with distilled water and then disrupted by six successive cycles of freezing and thawing. The material was centrifuged at 3,000 g at 4°C for 30 min. The sediment was resuspended in 4 ml PBS and was sonicated (B. Braun Sonifier, Germany) at constant power on high setting with repeated duty cycles per 0.3 s for 5 min for three cycles in ice bath of 4°C. The disruption was confirmed by light microscopic examination. The sonicated material was then centrifuged at 15,000 rpm at 4°C for 30 min. The supernatant fluid was taken as TSA and stored at −20°C until use.

Protein estimation

The protein content of TSA as determined by the Lowry et al. (1951) method was 0.63 mg/ml.

Conjugate

Commercially available horseradish peroxidase conjugated goat anti-human IgG (Genbio, Taiwan) was used for the ELISA.

Substrate

Commercially available substrate solution tetramethyl benzidine (TMB, Genbio, Taiwan) of 0.6 mg/ml of TMB in citric acid buffer plus 0.02% H₂O₂ was used.

Reference sera

The known positive and negative sera obtained from the reference laboratory (Dr. C. Sreekumar, Toxo laboratory, USDA, Maryland, USA) were aliquoted and stored at −20°C until use.

Standardization of in-house IgG ELISA by checker board titration

Optimum working dilution of antigen, sera and conjugate was determined by checker board titration using serial dilution of serum, antigen and conjugate. The sera were diluted as 1:50, 1:100, 1:200 and 1:400 and these were tested in a chequer board titration with known positive antigen serially diluted. The optimum dilution, which gave maximum difference between negative and positive antigen was selected as the working dilution of serum for use. Similarly, the antigen dilution was also standardized at various concentrations of 5, 10, 20 and 40 µg/ml. The conjugate dilutions of 1:500, 1:1,000, 1:5,000 and 1:10,000

were tested by chequer board titration with serially diluted known positive and negative sera and antigen. The optimum dilution, which gave the highest ratio between positive and negative control sample was selected. The sensitivity, specificity and positive and negative predictive value of the assay were calculated using ten known positive and negative reference sera.

Standardization of in-house IgG ELISA

The TSA was prepared and the protein concentration was determined which approximately amounts to 0.63 mg/ml. The antigen concentration was standardized at 10 µg/ml. The optimum dilution of serum and conjugate were found to be 1:100 and 1:1,000, respectively, by checker board titration.

Test procedure for in-house IgG Micro-ELISA

In-house IgG Micro-ELISA was performed as described by Voller et al. (1976) with minor modifications. The 96-well flat bottom polystyrene microtitre (ELISA) plate (Tarson products Pvt. Ltd., Kolkata, India) was recharged by filling up the wells with lukewarm water at least for 15 min. Thereafter the water was discarded by inverting and shaking the plate. The predetermined antigen solution (10 µg of TSA/ml) was prepared in 0.05 M Carbonate–bicarbonate buffer. Hundred µl of antigen was coated in the ELISA plate and incubated at 4°C for overnight. The plate was then washed with PBS-T for three times. Thereafter, 100 µl of 1% skimmed milk solution in PBS-T was added into the plate and incubated for 1 h at 37°C. The plate was then washed with PBS-T for three times. Then 100 µl of test sera prepared at 1:100 dilution was added to the plate. The positive and negative sera controls of same dilutions were added in the respective wells. The plate was incubated for one hour at 37°C. The plate was washed with PBS-T for three times. One hundred µl of 1:1,000 goat anti-human IgG HRP conjugate diluted in PBS-T was added on each well and incubated at 37°C for one hour. The plate was washed with PBS-T for three times. One hundred µl of TMB substrate solution was added in each well and incubated in a dark room at room temperature for 15 min and the reaction was stopped by adding 100 µl of 1 M sulphuric acid. The substrate and conjugate control was maintained in each plate. The optical density (OD) values were read in Multiscan ELISA reader (Biorad, France) at 450 nm reference filter.

The cut off value was calculated by adding mean OD value of two negative controls and mean OD value of three positive controls and the sum is divided by six. The test sera with absorbance values less than the cut off value were considered nonreactive and test sera with absorbance

values equal or greater than the cut off value were considered reactive. The cut off value for the IgG ELISA was found to be 0.144.

Comparison of ELISA with IFAT

The efficiency of the IgG ELISA was statistically compared for its test of significance by McNemar's test with our previous report on seroprevalence of toxoplasmosis using same set of samples by IgG IFAT (Sucilathangam et al. 2010).

Results

Seroprevalence of toxoplasmosis by ELISA

Out of 350 patients tested by in-house IgG ELISA, 46 patients (13.14%) had antibodies for toxoplasmosis with mean OD value of 0.2 ± 0.073 and the OD value ranged from 0.144 to 0.444. Among the immunocompetent group of 175 patients, 19 patients (10.86%) had antibodies to toxoplasmosis whereas, in immunodeficient group of 175 patients, 27 patients (15.43%) had antibodies for toxoplasmosis. There was no statistical difference ($P > 0.05$) between the immunocompetent and immunodeficient group (Table 1).

Out of 135 pregnant women tested, 13 (9.63%) were seropositive with mean OD value of 0.191 ± 0.069 (OD value ranged from 0.148 to 0.357). Among the 20 cases of lymphadenopathy, only three (15%) were seropositive with mean OD value of 0.162 ± 0.029 (OD value ranged from 0.144 to 0.196). Of the 20 ocular cases tested, only three (15%) were seropositive with mean OD value of 0.179 ± 0.043 (OD value ranged from 0.147 to 0.228).

Among the 160 HIV positive individuals tested by IgG ELISA, 24 (15%) were seropositive with mean OD value of 0.212 ± 0.079 (OD value ranged from 0.146 to 0.444). Out of 15 cases of malignancy, only three (20%) were seropositive with mean OD value of 0.173 ± 0.025 (OD value ranged from 0.146 to 0.196).

Comparison of IgG ELISA with IgG IFAT

The IgG ELISA test results were compared with our previous report on seroprevalence of toxoplasmosis using same set of samples by IgG IFAT (Sucilathangam et al. 2010). The overall seroprevalence of *T. gondii* in and around Tirunelveli district of Tamil Nadu was 11.71% based on IgG IFAT. Out of 350 patients tested by IgG IFAT, 41 patients (11.71%) had antibodies for *T. gondii* with mean IFAT titre of 43.32 ± 58.7 and the titre ranged from 1:16 to 1:256. Among the immunocompetent group of

Table 1 Seroprevalence of Toxoplasmosis by IgG-ELISA

S. No.	Category of patients	Number of patients examined	Number of patients positive	Per cent positive (%)	Mean OD value with $\pm$ SE and range of positive patients
	Immunocompetent*				
1	Pregnant women	135	13	9.63	0.191 $\pm$ 0.069 (0.148 –0.357)
2	Lymphadenopathy	20	3	15.0	0.162 $\pm$ 0.029 (0.144 –0.196)
3	Ocular	20	3	15.0	0.179 $\pm$ 0.043 (0.147 –0.228)
	Immunodeficient*				
4	HIV positive individuals	160	24	15.0	0.212 $\pm$ 0.079 (0.146 –0.444)
5	Malignancy	15	3	20.0	0.173 $\pm$ 0.025 (0.146 –0.196)
	TOTAL	350	46	13.14	0.2 $\pm$ 0.073 (0.144–0.444)

* No statistical difference ($P > 0.05$) between the immunocompetent and immunodeficient group (Chi-square value $\chi^2 = 1.60$)

175 patients, 19 patients (10.86%) had antibodies to *T. gondii*, whereas in immunodeficient group of 175 patients, 22 patients (12.57%) had antibodies for *T. gondii*. The sensitivity, specificity, positive and negative predictive value of IgG IFAT in detecting toxoplasmosis was 80, 90, 88.89, and 81.81%, respectively.

IgG ELISA was detected 46 (13.14%) seropositive cases out of 350 patients tested and the sensitivity, specificity, positive and negative predictive value of IgG ELISA in detecting toxoplasmosis was 90, 100, 100 and 90.9%, respectively. The testing efficiency of the IgG ELISA was statistically compared for its test of significance by McNemar's test with IgG IFAT for detection of *T. gondii* antibodies. Statistically the testing efficiency of IgG ELISA in detecting toxoplasmosis was higher than IFAT ($P < 0.05$).

Discussion

To date, many serologic kits commercially available for diagnosis of *T. gondii* infection are based primarily on the use of whole extracts of tachyzoites (Beghetto et al. 2006). However, these extracts contained large amount of proteins and other macromolecules; each of them can influence the results of the test. Therefore, the pseudo positive concordance often happens in the test by these kits. The antigens used in serologic diagnosis are mostly prepared from tachyzoites collected either from cell cultures or from infected mice. Owing to the different sources or different collecting and purification methods, the quantity and quality of the antigens are often varied; thus, the diagnostic system developed with such antigens is different in efficiency in these commercial kits (Taylor et al. 1990). Moreover, defined quantity of antigens and number of sample to be tested is limited in the commercial test kits and they are too costly to procure. However, in the in-house test procedures, large quantity of diagnostic antigen

with good quality can be produced with native antigen and quite large number of samples can be tested with limited cost.

The in-house IgG ELISA for toxoplasmosis was standardised and the cut off value for the ELISA was found to be 0.144 in the present study. The sensitivity and specificity of ELISA in detecting toxoplasmosis was found to be 90 and 100%, respectively. This was in accordance with the findings of Shirley et al. (1982), who reported that ELISA was more specific (100%).

The overall seroprevalence of toxoplasmosis in and around Tirunelveli district of Tamil Nadu was found to be 13.14% based on IgG ELISA in contrast with lower results obtained in a study conducted in Chandigarh, in which they have reported the overall prevalence of 5.4 and 4.66%, IgM and IgG anti-*Toxoplasma* antibodies respectively (Mohan et al. 2002).

In India, the exact seroprevalence of *T. gondii* is not known. However, using various diagnostic tests, the prevalence has been reported to be as low as 1% and as high as 80% in adults (Singh 2003). Until recently, the prevalence of *T. gondii* in the general population of India was considered to be low when compared with Western countries (Bowerman 1991; Mittal et al. 1995). This may be partially true, however, the data are based on samples obtained from patients admitted to hospitals in big cities. Because most of the population of India lives in rural villages with little means to get to city hospitals, the data are not reflective of the population as a whole. The type of serological tests used and the titres taken as evidence of infection are also important factors. However, only studies during 1990 indicated that the prevalence may be a lot higher than previously considered (Singh and Nautiyal 2004; Singh et al. 1994).

On the other hand, there are studies which have shown a higher prevalence than results in our study. In earlier studies, only 12% seropositivity for *T. gondii* was reported from South India (Bhatia et al. 1974), whereas, of late, a

high prevalence (75–77%) of *T. gondii* was reported from North India (Singh 2002). A study carried out in Jodhpur on seroprevalence of toxoplasmosis reported that *T. gondii* specific IgG and IgM antibodies assayed by ELISA were observed in 17.2% cases (Joshi et al. 1998). Another study performed by ELISA on the seroprevalence of *T. gondii* in Karnataka in 1,000 healthy adult population of voluntary blood donors, and reported the overall prevalence of 20.3% were positive for *T. gondii* IgG antibody, of which, 63% had high and 7% low avidity and 3.6% IgM positive (Sundar et al. 2007).

A study of seroprevalence of toxoplasmosis in general population and in HIV/AIDS patients in Bombay revealed that the overall seroprevalence was 30.9% (51/165) in the immunocompetent adult (34% in men and 26.2% in women). In HIV infected hosts the seroprevalence was 67.8% (Meisheri et al. 1997).

In the present study, among the immunocompetent group of 175 patients, 19 patients (10.86%) had antibodies to toxoplasmosis whereas, in immunodeficient group of 175 patients, 27 patients (15.43%) had antibodies for toxoplasmosis.

In our study, 13 (9.63%) were seropositive for *Toxoplasma gondii* in 135 pregnant women. High prevalence rate of anti-*Toxoplasma* antibody in pregnant women have been reported in recent studies. A study among primigravid women attending a secondary level hospital in a district of North India using IgG ELISA revealed 41.75% were seropositive for *Toxoplasma gondii* infection (Akoijam et al. 2002). A prospective study (Singh et al. 2004) on incidence and prevalence of toxoplasmosis in Indian pregnant women indicated that the overall IgG seroprevalence rate of toxoplasmosis was 45% by using IgG Avidity ELISA test. The prevalence of toxoplasmosis in pregnant women from Calcutta was reported as 7.1% whereas; it was 45% in pregnant women from New Delhi by using IgG Avidity ELISA test. (Mohan et al. 2002).

However, the knowledge about this infection, diagnosis and interpretation of the test results in pregnant women was a major problem in the Indian context (Singh 2003). Though, *Toxoplasma* infection does not cause repeated foetal losses, this is the most common indication for investigation of toxoplasmosis in India. There are some reports on the high prevalence rate of anti-*Toxoplasma* antibody in pregnant women with bad obstetric history (BOH). In this present study also, only three out of 19 (15.79%) cases with BOH were seropositive for toxoplasmosis. A study from Hyderabad, South India reported seroprevalence of toxoplasmosis was 33% (IgG) in lower socioeconomic group compared to higher socio-economic group (22%) in 236 women with BOH (Yashodhara et al. 2004). ELISA IgM and IgG antibodies for *Toxoplasma* were found in 20 and 55% of pregnant women with BOH

in Kumaon region respectively. (Naveen et al. 2005). The incidence of Torch infections in pregnancy wastage in women with BOH (14.66%) has been reported (Rajendra et al. 2006). Therefore, BOH alone was not to be taken as criteria for routine screening of toxoplasmosis.

In the present study, of the 20 ocular cases tested only three (15%) were seropositive. An outbreak of ocular toxoplasmosis occurred in Coimbatore, Tamil Nadu, in which 71.5% of particular area had high titres of both IgM and IgG antibodies, indicating that it was an acute infection, acquired through municipal water contamination (Manikandan et al. 2006).

Present study revealed that out of 15 cases of malignancy, only three (20%) was seropositive and 15% seropositive among the 160 HIV positive individuals tested by IgG ELISA whereas, a high seroprevalence rate of *T. gondii* of 67.8% in HIV patients was reported from Bombay (Meisheri et al. 1997).

The present study was designed to reveal the prevalence rate of anti-*Toxoplasma* antibody by using in-house IgG ELISA which was found to be 90% sensitivity and 100% specificity. Since in-house assays were cost effective and can be used for routine serological screening. The present study has also highlighted an overall 13.14% of seroprevalence of *T. gondii*, which constitutes 15.43% in immunocompromised and 10.86% in immunocompetent patients in and around Tirunelveli district of Tamil Nadu. The study underlines the importance of screening of this parasite especially in the immunocompromised patients.

The seroprevalence of *T. gondii* in pregnant women was about 9.63%, which necessitates initial screening for IgG antibodies and then a paired sera sample after 3 weeks should be tested for rise in titre. Thereafter, the IgM antibodies should be tested to exclude the recent infection. All seropositive women who seroconvert during pregnancy should be monitored and their amniotic fluid/foetus should be screened by PCR and Ultrasound, respectively to assess the infection.

Our recent previous report on seroprevalence toxoplasmosis in same districts of Tamil Nadu by in-house IFAT revealed 11.71% (Sucilathangam et al. 2010) whereas in the present study, the prevalence was 13.14% by in-house IgG ELISA by using same set of samples. The in-house IgG ELISA was found to be 90% sensitivity and 100% specificity. Statistically the testing efficiency of IgG ELISA in detecting toxoplasmosis was higher ($P < 0.05$) than IFAT. Therefore, IgG ELISA detected more number of seropositive cases than IFAT. The antigen for the IFAT is whole, killed tachyzoites affixed to microscope slides. The IFAT requires use of a fluorescence microscope. The toxoplasmosis enzymatic assay seems thus to be an excellent alternative to IF tests. Enzyme immunoassays are inherently attractive as they are sensitive, give only

objective results, and are suitable for large-scale automation. For routine serology, obtaining results by mere test observation of developed color, or even by automatic spectrophotometry and registration, certainly represents an advantage over fluorescence microscopy.

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